Reference: *Biol. Bull.*, 143: 506–512. (December, 1972)

THE INORGANIC COMPOSITION OF MOLLUSCAN EXTRAPALLIAL FLUID

MILES A. CRENSHAW

Forsyth Dental Center, Boston, Massachusetts and Dental Research Center, University of North Carolina, Chapel Hill, North Carolina

The molluscan extrapallial fluid is enclosed between the inner surface of the shell and the mantle, and it is analogous to the extracellular fluid of vertebrate bone. The determination of the compositions of these skeletal extracellular fluids would give better insight into the conditions under which calcification occurs.

It has been shown that extracellular fluid of bone is separated from other extracellular fluids (Neuman, 1969), and that it has a much higher potassium concentration (Triffit, Terepka and Neuman, 1968). This fluid is very diffuse; therefore, direct analysis is difficult. Although the volume of molluscan extrapallial fluid is very small, it is more readily accessible for direct analysis.

While some studies have been made of the bone fluid, little is known of the composition of the extrapallial fluid (Wilbur, 1964). De Waele (1930), using *Anodonta cygnea*, found that the composition of the extrapallial fluid was similar to the blood. Later, Florkin and Besson (1935) demonstrated that the extrapallial fluid of this freshwater clam was a fluid compartment separated from blood.

This is a report of the inorganic composition of the extrapallial fluids of three marine bivalves: *Mercenaria mercenaria, Crassostrea virginica* and *Mytilus edulis.*

Materials and Methods

The molluscs used in this study were collected from Cape Ann to Cape Cod, Massachusetts, and at Beaufort, North Carolina, throughout the year. They were maintained in aerated sea water at 21–23° and fed cultures of *Skeletonema costatum* and *Thalassiosira fluviatilis* twice weekly. (The original cultures of these diatoms were kindly furnished by Dr. R. R. L. Guillard, Woods Hole Oceanographic Institution.) Under these conditions the molluscs deposited new shell year-round as evidenced by increases of reduced weight in sea water.

Access to the extrapallial fluid was gained by drilling a hole through the center of one valve with a round dental burr. A stream of air was directed into the hole during the drilling to remove the powdered shell, and for cooling. The drilling was stopped just as the front edge of the burr penetrated the inner shell surface. A glass capillary was cemented into the hole, a short length of polyvinyl tubing fitted over the capillary, and the tubing sealed. Each animal was returned to an aquarium for at least two days before any samples were taken.

The animal was placed in a plastic dish containing two liters of Millipore (0.45 μ pore diameter) filtered sea water before the sample was taken. A sample of the sea water was taken just after sampling the extrapallial fluid. The sea water was aerated throughout this period. Samples were taken from oysters only when the

valves were closed because the extrapallial cavity of this animal is in direct contact with sea water when the valves are open. Samples from the other molluscs were taken at random with respect to the opening and closing of the valves. The integrity of the mantle and the seal around the capillary was checked after the samples were taken by injecting Evans blue into the extrapallial cavity and assaying for the dye in the sea water spectrophotometrically. When any evidence of leakage was found, the samples collected for that animal were discarded. Histological examination of a number of mantles indicated that only those from which the dye leaked from the extrapallial cavity were damaged.

Total CO_2 (free and dissolved CO_2 and the salts of carbonic acid) was determined by the Conway method (Conway, 1962). These samples were taken from the proximal end of the catheter with a microliter syringe and transferred directly into the diffusion dish without being exposed to air.

The pH was determined with a combination microprobe electrode cemented into the cap of a 2-ml plastic beaker with a conical bottom. An inlet for the sample was provided by cementing a short length of capillary tubing into a hole drilled through the bottom of the beaker. This arrangement allowed a precise determination of pH on 0.1 to 0.3 ml of extrapallial fluid. Samples were taken with a tuberculin syringe fitted with a needle and then forced into the beaker.

Samples for the other analyses were taken with a microliter syringe and immediatelly diluted for these analyses. It was found that a delay of more than 10 minutes between the collection and the dilution of the extrapallial fluid samples caused them to become very cloudy. Attempts to clarify these samples by centrifugation or by pressure or vacuum filtration were only partially successful. The values obtained for calcium on the partially clarified samples were lower than on the samples diluted shortly after their collection. For these reasons, the data reported here include only those samples diluted for analyses within 5 minutes after collection.

In situ measurements of the pH of the extrapallial fluid were made in the following manner. A 5-mm hole was drilled through the shell with a trephine, a 1-cm length of 5 mm (i.d.) glass tubing was cemented over the hole, and the combination pH electrode was inserted so that the tip was even with the inner shell surface. After the extrapallial fluid filled the glass tubing, the electrode was sealed to the glass tubing with a short length of plastic tubing. The animal was then returned to sea water, and the pH of the extrapallial fluid was measured at intervals over a 6 to 12 hour period. At the end of the experiment the electrode was removed, and the integrity of the mantle was checked with Evans blue and by histological examination.

The pooled samples of mantle fluid (the fluid between the two mantles), blood plasma, and extrapallial fluid from *M. mercenaria* were obtained from 24 animals in the following manner. The mantle fluid was collected by prying the valves apart a few millimeters, and allowing the fluid to drain into a beaker. The adductor muscles were then cut, the animal blotted, and the extrapallial fluid collected by teasing the mantles away from the valves. The animal was blotted again, and the blood was collected as it drained from an incision in the foot and then centrifuged.

Calcium and magnesium were determined with a Perkin-Elmer Model 303 or a 290B atomic absorption spectrophotometer. The final dilutions of the samples and standards for Ca determination contained 1% (w/v) lanthanum chloride. Sodium

and potassium were determined with a Coleman Model 12 flame photometer using a lithium internal standard. Chloride was determined with a Cotlove titrator. Sulfate was estimated by conductiometric titrations with $BaCl_2$ in 10 mN HCl.

Protein was determined by the method of Nayyar and Glick (1954) using bovine serum albumin to construct the standard curve. Hexosamine was determined according to Swann and Balazs (1962) using glucosamine as the standard. Ester sulfate was hydrolyzed by refluxing in 10 N HCl overnight. The liberated sulfate was then estimated with the barium chloranilate procedure of Wainer and Koch (1962).

Results

The average inorganic compositions of the extrapallial fluids from the three molluscs are shown in Table I. The magnesium concentrations are consistently 8% greater than would be expected from standard sea water analysis (Riley and Skirrow, 1965). This overestimation resulted from the failure to include a lanthanum salt in samples (Slavin, 1968).

Table I

Average inorganic composition of extrapallial fluids

Animal	n	Na (mEq l)	K (mEq l)	Ca (mEq l)	Mg (mEq l)	Cl (mEq l)	SO_4 (mEq l)	CO_2 (mM)	pH
Mercenaria mercenaria	24*	444	9.6	23.6	120	472	46.1	5.2	7.33
		±9**	±0.8	±2.0	±10	±8	±5.1	±1.9	±0.15
Crassostrea virginica	13	441	9.4	21.5	114	480	48.3	5.0	7.41
		±9	±0.5	±1.7	±6	±9	±2.3	±0.8	±0.16
Mytilus edulis	10	442	9.5	21.3	116	477	47.3	4.2	7.39
		±10	±0.5	±1.2	±6	±8	±2.3	±0.5	±0.17
Sea Water	47	427	9.0	18.5	106	496	51.1	2.5	7.91
		±9	±0.1	±0.4	±5	±6	±2.6	±0.1	±0.11

* Number of individual determinations.
** Mean ± standard deviation.

The total cation concentration in the extrapallial fluids was always greater than the total anion concentration. This difference was due to the presence of anions, such as phosphate and succinate, which were not determined.

The Donnan ratio for each ion was calculated for each extrapallial fluid sample. Except for calcium, the Donnan ratios for the individual ions were almost identical within a species (1.05 for *M. mercenaria*, 1.03 for *C. virginica* and 1.04 for *M. edulis*). The higher values for the calcium Donnan ratios indicate that 3.2 mEq/l Ca was bound in the *M. mercenaria* extrapallial fluid, 2.0 mEq/l in *C. virginica* and 1.9 mEq/l in *M. edulis*.

The pH of the extrapallial fluid from each animal was well below that of sea water. When extrapallial fluid samples were allowed to remain in the electrode vessel up to one hour, the pH rose steadily and the samples become cloudy and viscous. After exposure to room air for one hour, the maximum pH recorded was 7.6 to 7.7, which was probably due to the loss of CO_2 from the samples. Since this

pH change upon exposure to air was noted, the pH of *M. mercenaria* and *M. edulis* extrapallial fluids were measured *in situ*. The pH values obtained were within the ranges of those obtained by the sampling method. The pH of the extrapallial fluid of a mollusc decreased when the animal closed its valves and rose again when the valves were opened.

The calcium concentration in the extrapallial fluid also changed with the opening and closing of the valves. In eight specimens of *M. mercenaria*, the calcium concentration in the extrapallial fluid was 21.1 ± 1.8 (mean ± one standard deviation) and the pH was 7.41 ± 0.10 when the valves had been open for 10 minutes or longer. When the valves were closed for 15 minutes or longer, the calcium rose to 24.6 ± 2.1 mM, and the pH dropped to 7.25 ± 0.14.

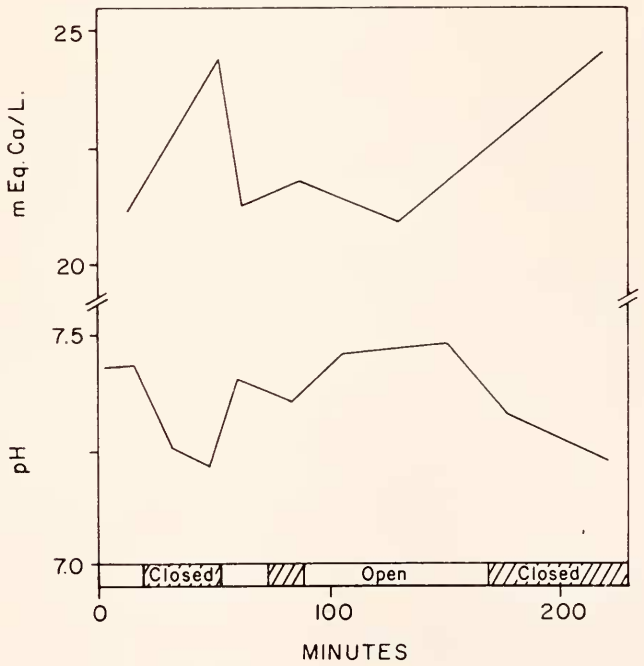

FIGURE 1. Calcium concentration and pH in the extrapallial fluid of *M. mercenaria* during the ventilation cycle. The hatched bars at the bottom of the graph indicate the periods when the valves were closed. The open bars show when they were open.

In two of the eight specimens of *M. mercenaria* and one of the six specimens of *M. edulis* examined, the minimum pH values were quite low (7.0 to 7.2). The valves of these animals remained closed throughout the period of observation. When the electrode was removed it was covered with crystalline calcium carbonate in each of these cases.

The correlation between the ventilation cycle and changes in the pH and calcium concentration in the extrapallial fluid was investigated further using *M. mercenaria*. The calcium concentration was determined in the extrapallial fluid from one side, and the pH was determined *in situ* from the other side. The data obtained from one animal are illustrated in Figure 1. The results confirmed the pre-

TABLE II

Composition of body fluids from M. mercenaria *before and after dialysis with sea water*

Fluid	Na (mEq/l)	K (mEq/l)	Ca (mEq/l)	Mg (mEq/l)	Cl (mEq/l)
	Original fluid				
Blood plasma	423	10.7	21.0	102	491
Extrapallial fluid	441	9.2	22.8	114	478
Mantle fluid	432	9.0	19.0	108	494
Sea water	427	9.0	18.5	106	496
	After dialysis				
Blood plasma	426	9.3	19.3	108	490
Extrapallial fluid	427	9.0	20.3	110	492
Mantle fluid	427	9.0	18.9	109	496

vious observations with respect to the ventilation cycle. The maximum pH observed in this series of experiments was below that observed when the extrapallial fluid samples were exposed to room air.

Calcium binding in the extrapallial fluid of *M. mercenaria* was investigated more thoroughly. In this experiment, the blood plasma and mantle fluid were compared. Table II shows the compositions of the three fluids before and after dialysis for 48 hours against 100 volumes of sea water at 4° C. The composition of the pooled extrapallial fluid was quite different from that of the pooled blood plasma, with calcium being the principal ion bound in fresh extrapallial fluid and potassium the primary ion bound in fresh blood. The mantle fluid was not different from sea water.

The Donnan ratios of the ions, except for potassium in the blood plasma and calcium in the extrapallial fluid, were reduced to unity by dialysis against sea water. This experiment demonstrated that the binding of these ions was done by a non-dialyzable fraction. The selective binding of calcium by this fraction *in vivo* appeared to be characteristic of the macromolecular components of the extrapallial fluid.

TABLE III

Composition of non-dialyzable material in the body fluids of M. mercenaria

	Impermeate* (mg/ml**)	Protein (mg/ml)	Mucopolysaccharide (mg/ml)
Blood plasma	4.25	2.14	1.27
Extrapallial fluid	3.68	0.34	3.22
Mantle fluid	1.42	0.04	0.23

* Material remaining in the dialysis tube.

** Concentration based on original volume of fluid.

Analyses of the non-dialyzable material (the impermeate) in each of these fluids were undertaken. Samples were exhaustively dialyzed against distilled water, freeze-dried, and weighed. The weights are shown in Table III.

An analysis for protein showed that 50% of the blood plasma impermeate was protein (Table III). This amount of protein could have accounted for the ionic gradients across the dialysis tubing shown in Table II. Protein accounted for less than 10% of the impermeate from extrapallial fluid.

An acid mucopolysaccharide fraction was prepared from each impermeate according to the method of Meyer, Linker, Davidson and Weismann (1953). The amounts obtained are shown in the last column of Table III. Analyses for ester sulfate and hexosamine showed that the SO_4/hexosamine ratio in the acid mucopolysaccharide fraction was 0.9 to 1.1.

Discussion

The inorganic composition of the extrapallial fluids of the three molluscs used in this study was different from that of sea water. The magnitude of the Donnan ratios for the individual ions, except for calcium, agrees with the low potentials measured across the isolated mantles of marine molluscs (C. Sterns and L. B. Kirschner, personal communications, cited by Wilbur, 1964).

In comparing extrapallial fluid and blood plasma, the potassium concentration in the extrapallial fluid was lower and the calcium concentration was higher than that of the blood plasma. Three important facts were shown by these observations: first, that the extrapallial fluid is a fluid compartment separated from blood, as found by Florkin and Besson (1935); second, that the extrapallial fluid is not formed by leakage from or damage to the cells of the mantle; and third, that the skeletal extracellular fluid of molluscs is different from that of vertebrates in this respect.

The pH values reported here were in agreement with those reported by N. Watabe and S. Kobayashi (personal communication, cited by Wilbur, 1964). The fluctuation in the pH and calcium concentration of the extrapallial fluid of an animal was associated with the ventilation cycle, with the pH falling and the calcium concentration rising when the valves closed. This is not unexpected, since the mollusc becomes anaerobic when the valves close, and previously deposited shell is dissolved to neutralize the succinic acid produced during this period (Crenshaw and Neff, 1969). Our calculations showed that a 100-g clam mobilized 2 mg shell per hour, and is large compared to the net shell formation. Thus, this turnover would mask changes in extrapallial fluid associated with shell formation, and makes estimates of shell growth using calcium-45, such as those done by Wilbur and Jodrey (1952), of questionable value.

The deposition of shell mineral on the pH electrode by some of the animals could have been normal shell formation. On the other hand, it may also have been an expression of the instability of the extrapallial fluid. However, the deposition of calcium carbonate at pH 7.0 to 7.2 was surprising.

The calcium concentration at the inner shell surface is above that of the external medium because the extrapallial fluid contains bound calcium. This binding is probably accomplished by a glycoprotein since mucopolysaccharides alone appear to have no selectivity in cation binding (Woodward and Davidson, 1968).

The bound calcium may have one of three functions. It may serve as a reservoir for calcium ions used in shell mineral formation, it may represent the dissolution of previously deposited shell (Crenshaw and Neff, 1969), or it may, with its binding agent, be a preliminary step in shell formation in which the calcium-glycoprotein complex undergoes further modification at the shell surface (Bevelander and Nakahara, 1969; Crenshaw, 1972).

I would like to thank Mrs. Jo Goulson for her aid during the preparation of this manuscript and Dr. Karl M. Wilbur for review and helpful criticism. This work was supported by USPHS Grants DE-00876 and DE-02668.

Summary

The inorganic composition of the extrapallial fluids of *Mercenaria mercenaria, Mytilus edulis* and *Crassostrea virginica* was significantly different from sea water.

Calcium was the principal ion bound in the extrapallial fluids.

This binding was accomplished by a non-dialyzable component that appeared to be a glycoprotein.

LITERATURE CITED

Bevelander, G., and H. Nakahara, 1969. An electron microscope study of the formation of the nacreous layer in the shell of certain bivalve molluscs. *Calc. Tiss. Res.*, **3**: 84–92.

Conway, E. J., 1962. *Microdiffusion Analysis and Volumetric Error.* [5th Ed.] Crosby Lockwood and Son, London, 467 pp.

Crenshaw, M. A., 1972. The soluble matrix from *Mercenaria mercenaria. Biomineralization*, in press.

Crenshaw, M. A., and J. M. Neff, 1969. Decalcification at the mantle-shell interface in molluscs. *Amer. Zool.*, **9**, 881–885.

De Waele, A., 1930. Le sang d'*Anodonta cygnea* et la formation de la coquille. *Mem. Acad. Roy. Belg. Classe Sci., (2)* **10**: 1–52.

Florkin, M., and G. Besson, 1935. Le liquid extrapalleal de l'*Anodonta* n'est pas identique au sang de cet animal. *C. R. Soc. Biol. (Paris)*, **118**: 1222–1224.

Meyer, K., A. Linker, E. A. Davidson and B. Weissmann, 1953. The mucopolysaccharides of bovine cornea. *J. Biol. Chem.*, **205**: 611–616.

Nayyar, S. N., and D. Glick, 1954. Studies in histochemistry XXXI. A method for the determination of protein in millimicrogram quantities. *J. Histochem. Cytochem.*, **2**: 282–290.

Neuman, W. F., 1969. The *milieu interieur* of bone: Claude Bernard revisited. *Fed. Proc.*, **28**: 1846–1850.

Riley, J. P., and G. Skirrow, Eds., 1965. *Chemical Oceanography, Vol. 1*, Academic Press, New York, 712 pp.

Slavin, W., 1968. *Atomic Absorption Spectroscopy*, John Wiley and Sons, New York, 307 pp.

Swann, D. A., and E. A. Balazs, 1966. Determination of the hexosamine content of macromolecules with manual and automated techniques using the *p*-dimethylbenzaldehyde reaction. *Biochim. Biophys. Acta*, **130**: 112–129.

Triffit, J. T., A. R. Terepka and W. F. Neuman, 1968. A comparative study of the exchange *in vivo* of major constituents of bone mineral. *Calc. Tiss. Res.*, **2**, 165–176.

Wainer, A., and A. L. Koch, 1962. Determination of inorganic sulfate in urine using barium chloranilate. *Anal. Biochem.*, **3**: 457–461.

Wilbur, K. M., and L. H. Jodrey, 1952. Studies on shell formation. I. Measurement of the rate of shell formation using Ca 45. *Biol. Bull.*, **102**: 269–276.

Woodward, C., and E. A. Davidson, 1968. Structure-function relationships of protein polysaccharide complexes: specific ion-binding properties. *Proc. Nat. Acad. Sci. Washington*, **60**: 201–205.